The *β2-tubulin* gene from three tephritid fruit fly species and use of its promoter for sperm marking

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ABSTRACT

To isolate testis-specific regulatory DNA that could be used in genetically transformed insect pest species to improve their biological control, *β2-tubulin* genes and their proximal genomic DNA were isolated from three economically important tephritid pest species, *Anastrepha suspensa*, *Anastrepha ludens*, and *Bactrocera dorsalis*. Gene isolation was first attempted by degenerate PCR on an *A. suspensa* adult male testes cDNA library, which fortuitously isolated the 2.85 kb *β1-tubulin* gene that encodes a 447 amino acid polypeptide. Subsequent PCR using 5′ and 3′ RACE generated the 1.4 kb *Asβ2-tubulin* gene that encodes a 446 amino acid polypeptide. Using primers to conserved sequences, the highly similar *A. ludens* and *B. dorsalis β2-tubulin* genes, encoding identical amino acid sequences, were then isolated. To verify *Asβ2-tubulin* gene identification based on gene expression, qRT-PCR showed that *Asβ2-tubulin* transcript was most abundant in pupal and adult males, and specific to the testes. This was further tested in transformants having the DsRed.T3 reporter gene regulated by the *Asβ2-tubulin* 1.3 kb promoter region. Fluorescent protein was specifically expressed in testes from third instar larvae to adults, and fluorescent sperm could be detected in the spermathecae of non-transgenic females mated to transgenic males.To confirm these matings, a PCR protocol was developed specific to the transgenic sperm DNA.

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1. Introduction

The ability to create transgenic strains of economically and medically important insect species has the potential to greatly enhance our ability to improve existing biological control methods and develop more novel means of control. An important facet of this technology is the use of sex- and tissue-specific regulatory systems for directed gene expression (Handler, 2002). In particular, a testis or spermatocyte-specific promoter that directs gene expression specifically in male gonadal tissue has been recognized as a means to improve the existing sterile insect technique (SIT) by facilitating genetic sexing, male sterility, and sperm marking. A visible fluorescent marker expressed in spermatocytes can be used for larval male selection, identification of mated females in the field, and for sperm precedence studies. Lethal gene expression similarly directed by a testis-specific promoter could confer male sterility that would provide a major advance over radiation-induced sterility. A primary candidate for testis-specific promoter regulation comes from the *β2-tubulin* gene. This gene was first identified in *Drosophila melanogaster* as functioning solely during

spermatogenesis during larval development and continuing throughout male adulthood. The *β2-tubulin* isoform is first observed in early spermatocytes, as a switch from *β1-tubulin* isoform production (Buttgereit and Renkawitz-Pohl, 1993), where it is specifically used in the axoneme for motile sperm development (Hoyle et al., 1995). This function has been elucidated, in part, by *Drosophila β2-tubulin* mutations that result in aberrant axonemal microtubules that disrupt sperm motility, with resultant sterility (Kemphues et al., 1982; Rudolph et al., 1987). Axoneme function is dependent, in particular, on a C-terminal tail amino acid motif specific to the *β2-tubulin* isoform whose variable nucleotide sequence distinguishes *β2-tubulin* from other members of the conserved tubulin family (Raff et al., 2008).

After *Drosophila*, the *β2-tubulin* gene was first isolated from a testes cDNA library from the moth *Heliothis virescens* (Davis and Miller, 1988) and more recently identified in the mosquitoes *Anopheles stephensi* (Catteruccia et al., 2005) and *Aedes aegypti* (Smith et al., 2007), and the tephritid fly, *Ceratitis capitata* (Scolari et al., 2008). In the latter three species the 5′ upstream promoter was linked to fluorescent protein genes that resulted in fluorescent sperm that could be detected in the testes, and for *A. aegypti* and *C. capitata*, in the spermathecae of females mated to transgenic males. The transparent nature of mosquito larvae allowed fluorescent testes to be identified in third instar males, with efficient sexing of

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A. stephensi demonstrated using an automated fluorescent-sorting system (Catteruccia et al., 2005).

Here we describe the isolation of the *β2-tubulin* gene from three tephritid fruit fly species, the Caribbean fruit fly, *Anastrepha suspensa*, the Mexican fruit fly, *Anastrepha ludens*, and the oriental fruit fly, *Bactrocera dorsalis*. Linking the promoter of the *A. suspensa β2-tubulin* (*Asβ2tub*) to the DsRed.T3 fluorescent protein (Bevis and Glick, 2002) allowed characterization of the gene by the tissue and developmental specificity of its expression. Initial efforts to isolate the *β2-tubulin* gene from *A. suspensa* resulted in the fortuitous isolation of the closely related *β1-tubulin* gene, allowing structural and developmental comparisons.

2. Materials and methods

2.1. Fruit fly rearing and nucleic acid preparation

A wild-type laboratory colony of *A. suspensa* (Homestead, Florida) and transgenic strains created from this colony were maintained at 25 °C in a larval diet of wheat germ-yeast-glucose. Third instar larvae were transferred to vermiculite with pupae maintained under humid conditions until adult emergence, and adults maintained on a yeast-sucrose diet. Pupal samples were provided from wild type strains of *A. ludens* (Tapachula, Mexico), *B. dorsalis* (Kahuku, Hawaii), and *Bactrocera papayae* (Penang, Malaysia) and kept frozen at −80 °C until DNA extraction. Genomic DNA was isolated from pupae using the DNAzol reagent (Molecular Research Center) with the addition of RNase, and total RNA was isolated from indicated tissues using Trizol (Invitrogen).

2.2. Isolation of the Asβ2tub gene and regulatory sequences

Full length cDNA was generated from RNA isolated from testes of 4–5 day old wild-type *A. suspensa* adult males using the SMART cDNA synthesis kit (BD Biosciences). *Asβ2tub* was isolated by first performing 3′ RACE using a forward degenerate primer (AH307) based on *β2-tubulin* homology in the C-terminal amino acid motif conserved in several insect species and a CDS 3′ adapter primer (see Table 1 for primer sequences). The 5′ region of the gene was generated by 5′ RACE using the 5′ SMART IV adapter primer and the AH311 gene specific primer.

Genomic DNA sequences of *Asβ2tub* were generated using primers from the 5′ and 3′ end of the full length cDNA. The 5′ UTR and promoter of *Asβ2tub*, and subsequently for the other *β2-tubulin* genes, were isolated by inverse PCR according to Handler and Harrell (2001a) using primers AH335 and AH333. All DNA

Table 1
PCR primer sequences.

Primer	Sequence
AH307	5′-ATGTTYGAYGCNAAGAAYATGATGG-3′
AH311	5′-GGTACTGCTGATATTCCGATACCAAA-3′
AH313	5′-CGGTCCAAAGATAAGTTATCAATT-3′
AH314	5′-ATGCGCGAAATAGTACATATTCAA-3′
AH323	5′-ATGGATCCGCGCATATTGTAATGTTTCCGCTAAA-3′
AH324	5′-ATCTCGAGTCTGAAGCGTGCGTCAGTTT-3′
AH333	5′-GCTTGAATATGTACTATTTCGCGCAT-3′
AH335	5′-CCGTCCGGATAATTTTGTATT-3′
AH358	5′-CGGGCTTCTTGGCCATGTAGAT-3′
QAsβ2F2	5′-TACGGCAATGTTTAGGCGCAAA-3′
QAsβ2R2	5′-GTACTGCTGATATTCCGATACCA-3′
QH3F	5′-CGAATTTCACGCAAAGCCACTG-3′
QH3R	5′-GCTCGTAAATCTGCTCCTTCAACC-3′
QDTS5F	5′-CAATCGTTCCCTGAATATCAAGTG-3′
QDTS5R	5′-GCAATCACAACATAATCCTCACC-3′

amplifications were performed using Expand Long Template DNA polymerase (Roche) with products cloned into the pCR4 TOPO TA vector (Invitrogen) and subsequently sequenced using vector primers. Gene amplification cycling conditions included initial denaturation at 95 °C for 5 min followed by 40 cycles of 95 °C for 30 s, 55 °C for 30 s and 68 °C for 5 min and a final extension at 68 °C for 10 min.

2.3. Sequence analysis and comparisons

Nucleotide and amino acid sequence analysis and comparisons were performed using MegAlign (DNASTAR, Inc.) and GeneWorks 2.5 (Oxford Molecular Group) software, and BLASTP (Altschul et al., 1997). Sequence pair distances were determined with MegAlign from ClustalW multiple sequence alignments (Higgins et al., 1994).

2.4. Plasmid construction

The 5′ upstream genomic sequence of *Asβ2tub* was amplified using primers AH324 and AH323 having XhoI and BamHI restriction sites, respectively. After subcloning into TOPO vector, a 1.3 kb XhoI/BamHI fragment was isolated and ligated into the corresponding cloning sites upstream to DsRed.T3 within the pDsRed.T3-N1 vector (Bevis and Glick, 2002) to create pAsβ2tub/DsRed.T3-N1. The pAsβ2tub/DsRed.T3-N1 plasmid was digested with AflII, blunted and digested with BglII with the resulting 2.1 kb Aβ2tub/DsRed.T3 fragment ligated into the BglII and EcoRV sites within the *piggyBac* vector, pB[XLPUbEGFP] to create pB[XLPUbEGFP/Asβ2tub-DsRed.T3].

2.5. Transformation and fluorescent marker detection

The pB[XLPUbEGFP/Asβ2tub-DsRed.T3] vector was transformed into wild type *A. suspensa* and *C. capitata* host strains with phspBac helper using standard methods (see Handler and Harrell, 2001a). Transformants expressing EGFP were selected using a Leica MZFLIII fluorescence stereo-microscope with a FITC/RSGFP filter set (#HQ41001; Chroma), and testis-specific DsRed.T3 fluorescence was observed using the Texas Red filter set (#HQ41004; Chroma). The same filter sets were used to observe fluorescent sperm using an Olympus IX71 inverted fluorescence microscope, with sperm nuclei detected after staining with 4′-6-Diamidino-2-phenylindole (DAPI) using the UV blue excitation cube (Olympus U-MWB cube). Three independent G1 transgenic individuals were selected in *A. suspensa* with the number of vector integrations determined by Southern blot hybridization as described previously (Handler and Harrell, 2001a). Lines F2B and M5A had single integrations, and line F4B had two integrations (data not shown). Integration number was not determined for *C. capitata* transformants.

2.6. Quantitative real-time PCR

Total RNA was isolated from tissues with 1 μg used for cDNA synthesis using the iScript™ cDNA synthesis kit (BioRad) after DNase I (Invitrogen) treatment. Quantitative real-time PCR (qRT-PCR) was performed on diluted cDNA (1:10) using the iQ SYBR Green Supermix (BioRad) in a Chromo4™ real-time PCR detector (BioRad). PCR cycling included an initial denaturation at 95 °C for 3 min followed by 40 cycles of 95 °C for 15 s, 55 °C for 10 s and 72 °C for 10 s with a plate read at the end of each cycle. All reactions were performed in triplicate and three independent experiments were performed to estimate variation. Gene specific primers for *Asβ2tub* (QAsβ2F2 and QAsβ2R2 yielding a 120 bp product) were designed manually due to limited nucleotide variations between *Asβ1tub* and *Asβ2tub*. Primers for the *A. suspensa* histone 3 (AsHis3) reference

gene (QH3F and QH3R yielding an 83 bp product) were designed using Beacon Designer 7.0 software (BioRad). Amplified fragments were analyzed on a 2% gel and subsequently cloned into pCR4 TOPO vector and sequenced to confirm specificity of the amplifications.

Relative accumulation of *Asβ2tub* normalized against AsHis3 was determined from the Ct values as described by Pfaffl (2001). PCR efficiency of each reaction was calculated using the LinRegPCR software (Ramakers et al., 2003). Genomic copy number of *Asβ2tub* was estimated by qRT-PCR by absolute quantification according to Lee et al. (2008) using a known single copy gene (*AsPros26*) as reference (Nirmala et al., 2009). *Asβ2tub* was amplified using primers QAsβ2F2 and QAsβ2R2 and *AsPros26* was amplified using primers QDTS5F and QDTS5R. Reactions were performed in triplicate using the iQ SYBR green supermix. The copy concentration of *Asβ2tub* and *AsPros26* in a known quantity of genomic DNA was estimated from Ct values using standard curves. The *Asβ2tub* copy number was determined by dividing the copy concentration of *Asβ2tub* by *AsPros26*.

3. Results

3.1. β1-tubulin and β2-tubulin gene isolation from A. suspensa

Isolation of the β2-tubulin gene from *A. suspensa* was first attempted by PCR on an adult male testes cDNA library using degenerate primers to conserved sequences from the β1-tubulin and β2-tubulin isoforms. It was thought that enrichment for the β2-tubulin transcript in male testes would result in this being the favored amplified product. PCR-generated genomic sequences, however, indicated that the β1-tubulin isoform was first isolated based on the presence and position of a 1.5 kb 5′ intron sequence that is consistent with the 2.6 kb and 1.0 kb β1-tubulin introns of *D. melanogaster* (Michiels et al., 1987) and *A. aegypti* (Smith et al., 2007), respectively. This is in comparison to the 59 bp and 57 bp introns found in the β2-tubulin gene from these species (Michiels et al., 1987; Smith et al., 2007). The *A. suspensa* β1-tubulin gene (*Asβ1tub*; GB accession no. EU980443) is 2852 bp with a 1511 bp intervening sequence at nts 58–1568, encoding a 447 amino acid polypeptide.

To more specifically target the β2-tubulin gene, a degenerate forward primer (AH307) was designed to the internal MFDAKNMM amino acid sequence motif specific to β2-tubulins (Smith et al., 2007). This primer was used with a reverse 3′ cDNA adapter primer that yielded a 0.6 kb sequence including the 3′ UTR. The conceptual translation of the 3′ terminus of the reading frame yielded the EGEFDEDEEGGGDE C-terminal tail amino acid motif specific to the *Drosophila* β2-tubulin (Popodi et al., 2008). 5′ RACE was then performed using an internal 3′ reverse primer (AH311), just upstream to this sequence, with a 5′ cDNA adapter primer that generated a 1.8 kb sequence. Direct PCR of genomic DNA using primers to the 5′ UTR and 3′ UTR generated a complete genomic sequence, and inverse PCR yielded a 2.0 kb 5′ upstream sequence including the presumptive promoter region. The *Asβ2tub* gene is 1398 bp encoding a 446 amino acid polypeptide (Fig. 1; GenBank accession no. EU938671). Comparisons of the genomic and cDNA sequences revealed a 60 bp intron sequence at genomic nucleotides 392–451, that is consistent with the size and position of the intron in other insect β2-tubulin genes. Based on the 5′ RACE sequence, a 394 bp 5′ UTR is deduced. Unlike the *C. capitata* β2-tubulin 5′ UTR, comparison to the genomic sequence does not reveal an intron in this region. A qRT-PCR analysis indicated that *Asβ2tub* exists as a single copy gene per haploid genome, consistent with other identified β2-tubulins.

3.2. β2-tubulin gene isolation from A. ludens and B. dorsalis

Primers known to amplify the *Asβ2tub* sequence were then used in direct (AH314 and AH311) and inverse (AH335F and AH333R) PCR to isolate putative genomic β2-tubulin sequences from the Mexican fruit fly, *A. ludens*, and the oriental fruit fly, *B. dorsalis*, with intron sites determined by comparison to the *A. suspensa* cDNA and amino acid sequences. The *A. ludens* β2-tubulin (*Alβ2tub*) gene is 1396 bp with a putative 58 bp intron at nucleotides 394–451, encoding a 446 amino acid polypeptide (Fig. 1; GenBank accession no. EU938672). By comparison to the highly similar *A. suspensa* 5′ UTR, a 451 bp 5′ UTR is deduced.

The *B. dorsalis* β2-tubulin (*Bdβ2tub*) gene is 1405 bp with a putative 67 bp intron at nucleotides 394–460, encoding a 446 amino acid polypeptide (Fig. 1; GenBank accession no. EU938673). Amplifications from the papayae fruit fly, *B. papayae*, genomic DNA yielded sequences identical to the *Bdβ2tub*, that further suggest that these sibling species within the *B. dorsalis* species complex are conspecific.

3.3. β2-tubulin sequence comparisons

Consistent with other insect β2-tubulin genes, the tephritid β2-tubulin genes presented here are nearly identical to one another and with the *C. capitata* gene reported previously (Scolari et al., 2008). All share a single small intron having varying positions and length (58–67 bp), but based on conceptual translations they have the same amino acid sequence identical to the β2-tubulins in *C. capitata* (Scolari et al., 2008) and *D. melanogaster* (Michiels et al., 1987). This sequence diverges approximately 5% from the β2-
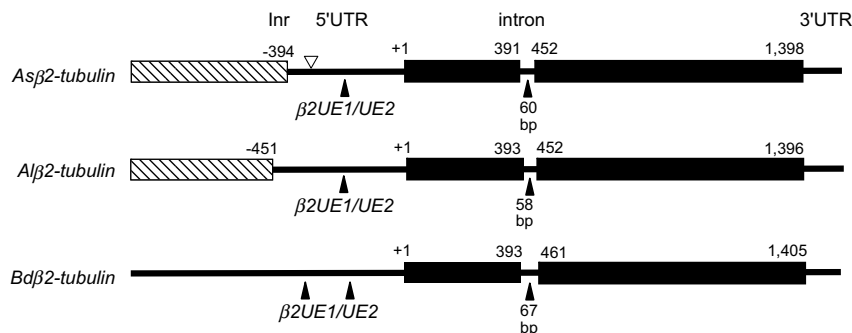
Fig. 1. Schematic diagram (not to scale) of the β2-tubulin gene sequences from *A. suspensa* (*Asβ2tub*), *A. ludens* (*Alβ2tub*), and *B. dorsalis* (*Bdβ2tub*). Each gene has two exons (black boxes) comprising a 1338 bp coding sequence (starting at +1) interrupted by a single intron. Designated upstream sequences include a partial region (∼500 bp) of the presumed promoter (striped box), transcription initiation site (Inr), and the 5′ UTR including putative *β2UE1/UE2* sites (see Fig. 3 for positions), with other non-coding sequences designated by a black bar. The Inr position for *Asβ2tub* is based on 5′ RACE, and inferred for *Alβ2tub* based on sequence homology to *Asβ2tub*, and was not determined for *Bdβ2tub* (and thus an upstream promoter is not designated). Based on homology, a 59 bp deletion in the *Asβ2tub* 5′ UTR relative to *Alβ2tub* is shown (open triangle).

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                    10        20        30        40        50        60        70
A. suspensa      MREIVHIQAGQCGNQIGGKFWEVISDEHCIDATGTYYGDSDLQERINVYYNEATGAKYVPRAILVDLEPGTMDS 75
A. ludens        MREIVHIQAGQCGNQIGGKFWEVISDEHCIDATGTYYGDSDLQERINVYYNEATGAKYVPRAILVDLEPGTMDS 75
B. dorsalis      MREIVHIQAGQCGNQIGGKFWEVISDEHCIDATGTYYGDSDLQERINVYYNEATGAKYVPRAILVDLEPGTMDS 75
C. capitata      MREIVHIQAGQCGNQIGGKFWEVISDEHCIDATGTYYGDSDLQERINVYYNEATGAKYVPRAILVDLEPGTMDS 75
D. melanogaster  MREIVHIQAGQCGNQIGGKFWEVISDEHCIDATGTYYGDSDLQERINVYYNEATGAKYVPRAILVDLEPGTMDS 75
A. aegypti       MREIVHIQAGQCGNQIGAKFWEVISDEHGIDATGAYGGDSDLQERINVYYNEATGGKYVPRAVLVDLEPGTMDS 75
B. mori          MREIVHIQAGQCGNQIGAKFWEVISDEHGIDATGAYSGDSDLQERINVYYNEASGGKYVPRAVMVDLEPGTMDS 75

                    80        90       100       110       120       130       140       150
A. suspensa      VRSGAFGQIFRPDNFVFGQSGAGNNWAKGHYTEGAELVDSVLDVVRKESEGCDCLQGFQLTHSLGGGTGSGMGTL 150
A. ludens        VRSGAFGQIFRPDNFVFGQSGAGNNWAKGHYTEGAELVDSVLDVVRKESEGCDCLQGFQLTHSLGGGTGSGMGTL 150
B. dorsalis      VRSGAFGQIFRPDNFVFGQSGAGNNWAKGHYTEGAELVDSVLDVVRKESEGCDCLQGFQLTHSLGGGTGSGMGTL 150
C. capitata      VRSGAFGQIFRPDNFVFGQSGAGNNWAKGHYTEGAELVDSVLDVVRKESEGCDCLQGFQLTHSLGGGTGSGMGTL 150
D. melanogaster  VRSGAFGQIFRPDNFVFGQSGAGNNWAKGHYTEGAELVDSVLDVVRKESEGCDCLQGFQLTHSLGGGTGSGMGTL 150
A. aegypti       VRAGPFGQIFRPDNFVFGQSGAGNNWAKGHYTEGAELVDSVLDVVRKESEGCDCLQGFQLTHSLGGGTGSGMGTL 150
B. mori          VRSGPFGQIFRPDNFVFGQSGAGNNWAKGHYTEGAELVDSVLDVVRKEAEGCDCLQGFQLTHSLGGGTGSGMGTL 150

                   160       170       180       190       200       210       220
A. suspensa      LISKIREEYPDRIMNTFSVVPSPKVSDTVVEPYNATLSVHQLVENTDETYCIDNEALYDICFRTLKLTTPTYGDL 225
A. ludens        LISKIREEYPDRIMNTFSVVPSPKVSDTVVEPYNATLSVHQLVENTDETYCIDNEALYDICFRTLKLTTPTYGDL 225
B. dorsalis      LISKIREEYPDRIMNTFSVVPSPKVSDTVVEPYNATLSVHQLVENTDETYCIDNEALYDICFRTLKLTTPTYGDL 225
C. capitata      LISKIREEYPDRIMNTFSVVPSPKVSDTVVEPYNATLSVHQLVENTDETYCIDNEALYDICFRTLKLTTPTYGDL 225
D. melanogaster  LISKIREEYPDRIMNTFSVVPSPKVSDTVVEPYNATLSVHQLVENTDETYCIDNEALYDICFRTLKLTTPTYGDL 225
A. aegypti       LISKIREEYPDRIMNTFSVVPSPKVSDTVVEPYNATLSVHQLVENTDESYCIDNEALYDICFRTLKLTTPTYGDL 225
B. mori          LISKIREEYPDRIMNTFSVVPSPKVSDTVVEPYNATLSVHQLVENTDETYCIDNEALYDICFRTLKLTTPTYGDL 225

                   230       240       250       260       270       280       290       300
A. suspensa      NHLVSATMSGVTTCLRFPGQLNADLRKLAVNMVPFPRLHFFMPGFAPLTSRGSQQYRALTVPELTQQMFDAKNMM 300
A. ludens        NHLVSATMSGVTTCLRFPGQLNADLRKLAVNMVPFPRLHFFMPGFAPLTSRGSQQYRALTVPELTQQMFDAKNMM 300
B. dorsalis      NHLVSATMSGVTTCLRFPGQLNADLRKLAVNMVPFPRLHFFMPGFAPLTSRGSQQYRALTVPELTQQMFDAKNMM 300
C. capitata      NHLVSATMSGVTTCLRFPGQLNADLRKLAVNMVPFPRLHFFMPGFAPLTSRGSQQYRALTVPELTQQMFDAKNMM 300
D. melanogaster  NHLVSATMSGVTTCLRFPGQLNADLRKLAVNMVPFPRLHFFMPGFAPLTSRGSQQYRALTVPELTQQMFDAKNMM 300
A. aegypti       NHLVSATMSGVTTCLRFPGQLNADLRKLAVNMVPFPRLHFFMTGFAPLTSRGSQQYRALTVPELTQQMFDAKNMM 300
B. mori          NHLVSATMSGVTTCLRFPGQLNADLRKLAVNMVPFPRLHFFMPGFAPLTSRGSQQYRALSVPELTQQMFDAKNMM 300

                   310       320       330       340       350       360       370
A. suspensa      AACDPRHGRYLTVAAIFRGRMSMKEVDEQMLNIQNKNSSFFVEWIPNNCKTAVCDIPPRGLKMSATFIGNSTAIQ 375
A. ludens        AACDPRHGRYLTVAAIFRGRMSMKEVDEQMLNIQNKNSSFFVEWIPNNCKTAVCDIPPRGLKMSATFIGNSTAIQ 375
B. dorsalis      AACDPRHGRYLTVAAIFRGRMSMKEVDEQMLNIQNKNSSFFVEWIPNNCKTAVCDIPPRGLKMSATFIGNSTAIQ 375
C. capitata      AACDPRHGRYLTVAAIFRGRMSMKEVDEQMLNIQNKNSSFFVEWIPNNCKTAVCDIPPRGLKMSATFIGNSTAIQ 375
D. melanogaster  AACDPRHGRYLTVAAIFRGRMSMKEVDEQMLNIQNKNSSFFVEWIPNNCKTAVCDIPPRGLKMSATFIGNSTAIQ 375
A. aegypti       AACDPRHGRYLTVAAIFRGRMSMKEVDEQMLNIQSKNSSYFVEWIPNNVKTAVCDIPPRGLKMSSTFIGNSTAIQ 375
B. mori          AACDPRHGRYLTVAAVFRGRMSMKEVDEQMLNIQNKNSSYFVEWIPNNVKTAVCDIPPRGLKMSATFIGNTTAIQ 375

                   380       390       400       410       420       430       440
A. suspensa      ELFKRVSEQFTAMFRRKAFLHWYTGEGMDEMEFTEAESNMNDLVSEYQQYQEATADEEGEFDEDEEGGGDE 446
A. ludens        ELFKRVSEQFTAMFRRKAFLHWYTGEGMDEMEFTEAESNMNDLVSEYQQYQEATADEEGEFDEDEEGGGDE 446
B. dorsalis      ELFKRVSEQFTAMFRRKAFLHWYTGEGMDEMEFTEAESNMNDLVSEYQQYQEATADEEGEFDEDEEGGGDE 446
C. capitata      ELFKRVSEQFTAMFRRKAFLHWYTGEGMDEMEFTEAESNMNDLVSEYQQYQEATADEEGEFDEDEEGGGDE 446
D. melanogaster  ELFKRVSEQFTAMFRRKAFLHWYTGEGMDEMEFTEAESNMNDLVSEYQQYQEATADEEGEFDEDEEGGGDE 446
A. aegypti       ETFKRIAEQFTAMFRRKAFLHWYTGEGMDEMEFTEAESNMNDLVSEYQQYQEATADEEGEFDEEEEGGEE 445
B. mori          ELFKRISEQFTAMFRRKAFLHWYTGEGMDEMEFTEAESNMNDLTSEYQQYQDATADDEGEFDEEAEEGLEE 446
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Fig. 2. ClustalW multiple sequence alignment of β2-tubulin amino acid sequences from *A. suspensa*, *A. ludens*, and *B. dorsalis*, *C. capitata* (GenBank accession no. EU386342), *D. melanogaster* (NP524290), *A. aegypti* (DQ833526), and *B. mori* (CAA52906). Amino acid positions for the consensus sequence are shown above the sequences, amino acids that differ from the consensus are boxed in black, and hyphens indicate introduced gaps. The underlined sequence represents the C-terminal tail motif specific to β2-tubulins.

tubulin amino acid sequences in *A. aegypti* (Smith et al., 2007) and *Bombyx mori* (Kawasaki et al., 2003) (Fig. 2).

Approximately 1 kb of 5′ DNA sequence upstream to the β2-tubulin translational start codon from the tephritid species was compared (see Supplementary Fig. 1). Consistent with their phylogenetic relationship, the *A. suspensa* and *A. ludens* upstream sequences, including the promoter region tested for function (see below), are nearly identical (>97% nucleotide identity) except for a 59 bp insertion within the *A. ludens* 5′ UTR at position −318 to −260 (Fig. 1). Pairwise comparisons show much lower identities of approximately 49–53% between the other tephritid β2-tubulin 5′ sequences, including the sequence from *C. capitata*, though discrete regions of high similarity exist. Despite the sequence divergence, we show below that the *A. suspensa* As*β2tub* promoter maintains heterologous function in *C. capitata* males.

Consistent with *D. melanogaster* (Santel et al., 2000) the *A. suspensa* sequence has an apparent TATA-less promoter with a putative transcription initiation site (Inr) having a CAGT core at position −394 (relative to the ATG translational start codon at +1). A transcriptional start site for *A. ludens* is inferred by sequence identity to *A. suspensa*, that would place its putative Inr site at −451. A lack of sequence identity precludes postulating the Inr position for *B. dorsalis*. Within the highly conserved 5′ UTRs is a putative β2UE1 14 bp activator element, necessary for cell-type specificity in

Drosophila (Michiels et al., 1989) and an adjacent putative β2UE2 enhancer element having only a positive quantitative influence in *Drosophila* (Michiels et al., 1993). These elements are upstream to the Inr in *Drosophila*, but location in the 5′ UTR is consistent with putative elements in other non-drosophilid dipterans (Smith et al., 2007; Scolari et al., 2008). The 28 bp sequences and positions for the β2UE1/β2UE2 putative elements are nearly identical in the Anastrepha species (Figs. 1 and 3). The exceptions are an initial 5′ adenosine in *A. suspensa* at −215, which is replaced by a guanidine in *A. ludens* at −216. The very highly conserved ATCG motif for β2UE1, does not otherwise exist within the 1.3 kb region upstream to their ATG translational start codons. A putative Inr cannot be deduced for the *B. dorsalis* genomic sequence, but putative β2UE1/β2UE2 sequences exist at −325 and −204.

3.4. As β2-tubulin gene expression

To verify the identification of As*β2tub* based on its gene expression, qRT-PCR was performed on cDNA generated from transcripts isolated from various developmental stages of male individuals. To identify larval males, we used a previously isolated *A. suspensa* transgenic strain having a Y-linked vector integration (pB[PUbDs-Red.T3]; Handler and Harrell, 2001b) that expresses the DsRed fluorescent protein specifically in males throughout development

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              β2UE1                        β2UE2        position
              _____                 _____
Dm β2t        ATCGT----AGTAGCCTATTTGTGAACATTC           -216
As β2t        ATCGTCTGCACTAG-AAATTT-T-AATTTTC
              ***** * *** ***** * ** ***

              _____                 _____
Dm β2t        ATCGT----AGTAGCCTATTTGTGAACATTC           -215
Al β2t        GTCGTCTGCACTAGAAATTT-T-AATTTTC
               ****   * ***  **** * ** ***

              _____              _____
Dm β2t        ATCGTAGTAGC-CTATTTGTGAACATTC             -325
Bd β2t_1      ATCGTTTTTTCAGAATTTTTTCACA--C
              *****  *  *   **** *  *** *

              _____                _____
Dm β2t        ATCGTAGTAGCCTATTTGTGAACAT-TC             -204
Bd β2t_2      ATCGAA--AGTGTA-CTAAGAAAACGTG
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Fig. 3. Sequence alignments showing putative β2UE1/β2UE2 sequences in the 5′ UTR of the tephritid β2-tubulin genes (A. suspensa, As β2t; A. ludens, Al β2t; and B. dorsalis, Bd β2t) based on similarity to the known sequences in D. melanogaster. Upstream 1.0 kb sequences for each tephritid gene were individually aligned to the Drosophila β2UE1/β2UE2 sequence (Dm β2t), with the highest identity sequences shown. Tephritid sequence positions are for the 5′ nucleotide relative to the upstream ATG translation start site (+1). Hyphens indicate introduced gaps and asterisks indicate identities.

Fig. 4. Histograms showing RNA transcript accumulation relative to AsHis3 for Asβ2-tubulin (black) and Asβ2tub-DsRed (gray) in indicated samples as assayed by qRT-PCR (see Materials and methods). In (A) relative transcript accumulation is shown for Asβ2-tubulin in transgenic male (Y, pB[PUbDsRed.T3]) sample preparations from embryos (E), second instar larvae (2L), early third instar larvae (E3L), late third instar larvae (L3L), early pupae (EP), late pupae (LP), 3 d adult males (M3d), and 8 d adult males (M8d). In (B) relative transcript accumulation of Asβ2-tubulin and Asβ2t-DsRed.T3 in the testes from three transgenic lines (F4B T, F2B T, M5A T) and whole adult females (F4B F) and male adult carcasses after dissection of the testes (F4B C) from line F4B are shown. Relative transcript levels in females and male carcasses are below 1 in all transgenic lines (data not shown for lines F2B and M5A).

(A.M. Handler, unpublished). Consistent with β2-tubulin expression in Drosophila, Asβ2tub transcript levels normalized to AsHis3 were barely detected in second instar and early third instar larvae, with low but significant increases in late third instar and early pupal stages (Fig. 4A). Seven to nine-fold increases were then observed in late pupal stage males, and in adults up to 8 days old.

Further expression analysis was achieved by linking 1.3 kb of the presumed Asβ2tub regulatory 5′ upstream sequence to a DsRed.T3 fluorescent protein reporter gene, that was transformed into a wild type A. suspensa host strain by piggyBac transformation. Transcript levels of the native Asβ2tub gene and the Asβ2tub-DsRed reporter contruct from whole females, male carcasses after removal of testes, and their dissected testes were then compared from three different transgenic lines (Fig. 4B). Relative transcript levels of the two genes remained at similar basal levels in females and male carcasses (below 1) in all three transgenic lines (only F4B is shown in Fig. 4B), while in testes both Asβ2tub and Asβ2tub-DsRed increased significantly. Compared to Asβ2tub, transcript levels of Asβ2tub-DsRed were 2.5 fold greater in lines F4B and M5A and 6.0 fold greater in line F2B. Taken together, the Asβ2tub transcript accumulation analysis showing sex-, tissue- and developmental specificity consistent with the Drosophila gene verified isolation of the A. suspensa cognate. The two to six-fold higher transcript levels of Asβ2tub-DsRed.T3 compared to Asβ2tub in the transgenic lines proves promoter function for the 5′ upstream sequence, and suggests that the Asβ2tub 3′ UTR, lacking in the transgene, is not essential for normal promoter activation, nor stabilization of a heterologous transcript, though an influence on Asβ2tub transcript stabilization remains to be evaluated. The unexpected relatively high level of Asβ2tub-DsRed.T3 transcript, especially in line F2B, is not understood, but might be due to a genomic enhancer effect related to the genomic insertion site, or relatively higher transcript stability.

3.5. Asβ2tub promoter function using the DsRed.T3 reporter

To further analyze Asβ2tub promoter function, reporter gene expression was visually observed in Asβ2tub-DsRed.T3 transgenic males. Consistent with expression of the Drosophila β2-tubulin isoform during spermatogenesis, red fluorescence was specific to larval and adult male testes, and spermatocytes extruded from the testes and sperm storage organs in mated females (Fig. 5). Red fluorescence was f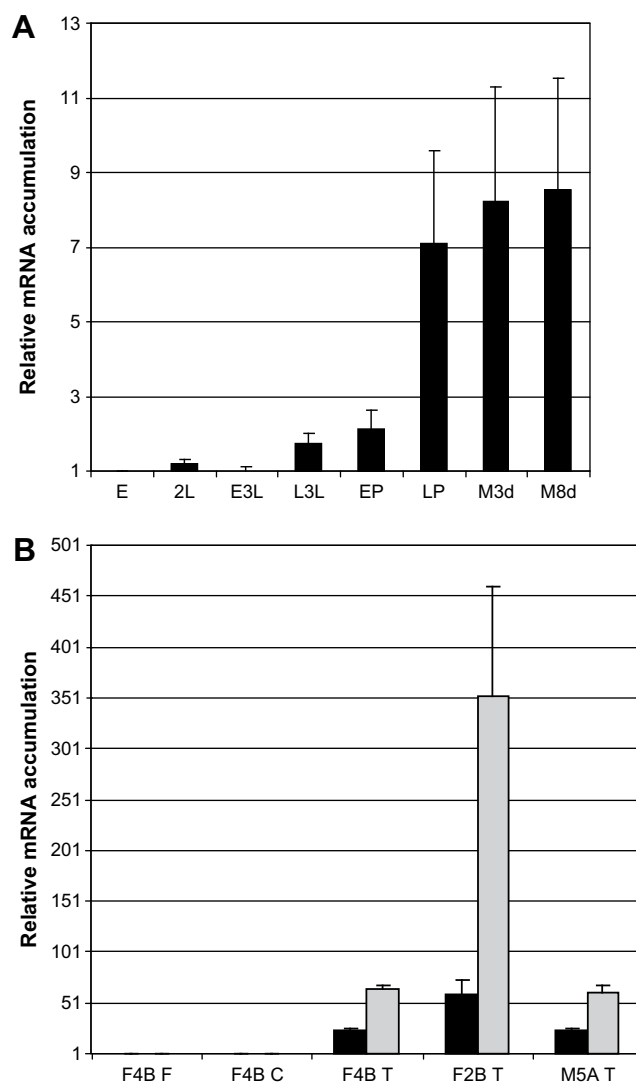irst detected through the external cuticle in the posterior region of third instar larvae, that later developed into adult males (Fig. 5a). Fluorescence could not be detected in undissected earlier stage larvae or in their dissected testes. Fluorescence was easily and consistently detected in the posterior abdomen of adult transgenic males, with the source of fluorescence verified after dissection of the testes (Fig. 5b,c). Fluorescence was further detected in sperm bundles from gently squashed testes, in addition to other unidentified seminal material (not shown).

3.6. Asβ2tub-DsRed for sperm marking

An important application for fluorescent-marked sperm is to determine if females trapped in the field have mated with released males, requiring sperm-specific markers. To assess the detection of fluorescent-marked sperm in mated females, non-transgenic and
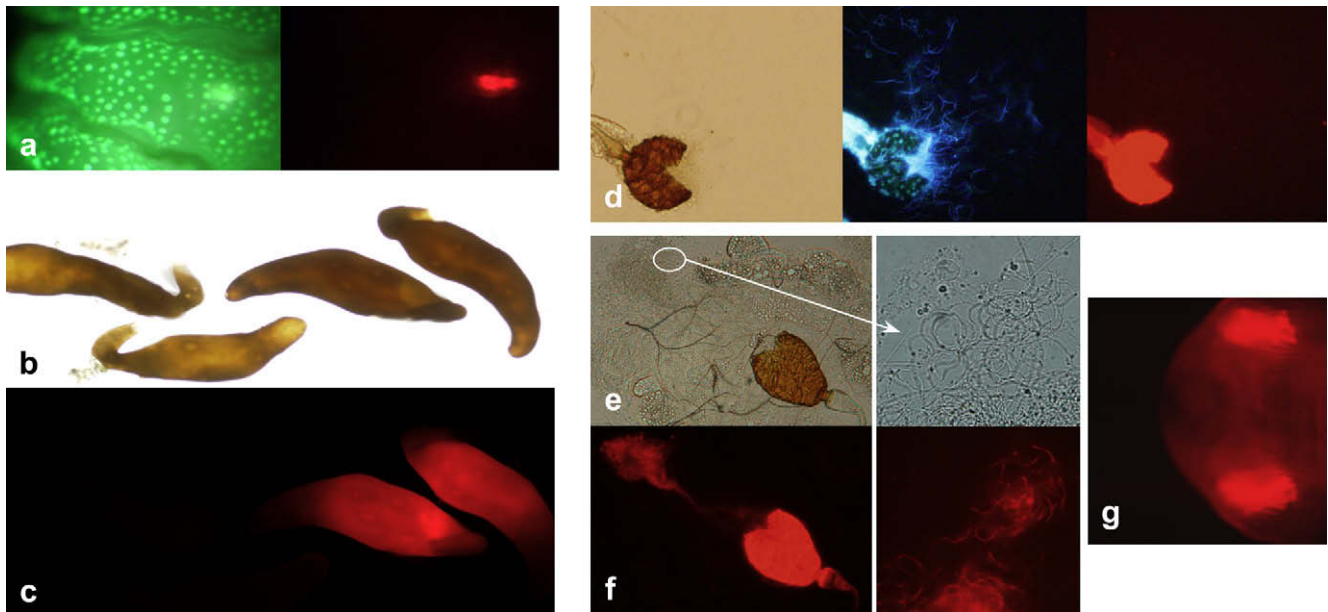
Fig. 5. Epifluorescent and brightfield images of testes from males transformed with pB[XLPUbEGFP/Asβ2tub-DsRed.T3], and seminal material from spermathecae dissected from non-transgenic females mated to non-transgenic or transgenic males. The posterior region of a transgenic *A. suspensa* third instar larval male (a) showing epidermal EGFP fluorescence (left) and testis-specific DsRed fluorescence (right); (b) testes from a non-transgenic adult male (left) and a transgenic male (right) under brightfield, and (c) DsRed epifluorescence showing fluorescence limited to the transgenic testes; (d) seminal material extruded from a gently squashed spermatheca from a non-transgenic female mated to a non-transgenic male under brightfield (left), DAPI staining (center), and DsRed epifluorescence (right) showing a lack of fluorescence in sperm detected by DAPI; (e) seminal material extruded from a spermatheca from a non-transgenic female mated to a transgenic male under brightfield with a region of exudate and sperm bundles (circled) observed at higher magnification (400×) in the right-hand panel (arrow); (f) the same images as in (e), but under DsRed epifluorescence showing fluorescence specific to the seminal material exudate, and sperm bundles under 400× magnification in the right-hand panel; and (g) posterior/ventral view of a *C. capitata* adult male abdomen transformed with the Asβ2tub-DsRed.T3 transgene under DsRed epifluorescence showing testis-specific fluorescence (verified after dissection; data not shown).

transgenic males were crossed to non-transgenic virgin females that were then inspected by dissection after mating. In control tests of non-transgenic males mated to non-transgenic females, sperm extruded from the spermathecae could be detected by DAPI staining, but these did not exhibit DsRed fluorescence (Fig. 5d). When transgenic males were mated, however, red fluorescent sperm could be detected in the spermathecal duct (not shown), as well as in sperm bundles and seminal material extruded from spermathecae after gentle squashing (Fig. 5e,f). The spermathecae themselves are highly autofluorescent from cuticular material, and the presence of sperm could not be determined without extrusion. Heterologous function for the *Asβ2tub* promoter was demonstrated by the strong expression of the DsRed.T3 reporter in the testes of *C. capitata* males transformed with the *Asβ2tub-DsRed.T3* reporter construct (Fig. 5g).

Since the spermathecae and stored sperm may degrade in trapped flies that have died, sperm-specific fluorescence may not be detectable or distinguished from autofluorescence due to necrosis. Thus a PCR assay for the *Asβ2tub-DsRed.T3* transgene was developed using primers AH313 and AH358, specific to the Asβ2tub promoter and DsRed.T3 sequences, respectively, which yield an 850 bp product. Wild type virgin females were mated to males heterozygous for the *Asβ2tub-DsRed.T3* transgene, with genomic DNA then extracted from individual abdomens of the females. PCR for the *Asβ2tub-DsRed.T3* transgene yielded varying amounts of the 850 bp product from each abdominal sample, though its presence was unambiguous and not observable in negative controls (Fig. 6).

4. Discussion

The testis-specific *β2-tubulin* gene and its 5′ upstream regulatory sequence region have been isolated and sequenced from three economically important tephritid fruit fly species, and the constitutive *β1-tubulin* gene has been isolated from *A. suspensa*.

Consistent with other *β2-tubulin* genes, there is a high level of amino acid conservation among the tephritid species and with other insects, as well as some nucleotide sequence conservation in the promoter region among the tephritids. Conservation of the amino acid sequence is quite strong, with identical polypeptides in all tephritid species, as well as *D. melanogaster*. Thus, the high β2-tubulin protein conservation originally observed among drosophilids extends to more distantly related dipterans (Michiels et al., 1987). Yet this does not extend to mosquitoes such as *A. aegypti* whose *β2-tubulin* (Smith et al., 2007) is 95% identical, and has a greater similarity to the sequence from *B. mori* (Kawasaki et al., 2003).

Putative *β2UE1/β2UE2* elements, shown to confer cell-type specificity in *Drosophila* (Michiels et al., 1989, 1993), have been identified in the three tephritid species. These sequences all reside in the 5′ UTR regions, which is consistent for putative *β2UE1/β2UE2* elements identified in other dipteran species. An AT-rich *β2DE1* element, shown to confer *β2-tubulin* transcript stabilization in *Drosophila* (Santel et al., 2000), was not identified in any of tephritid genes, though AT-rich regions do exist within the 5′ UTRs.

For *A. suspensa*, the *β2-tubulin* gene was identified and characterized by the sex, stage and tissue specificities of its transcription using qRT-PCR, which was limited to the testes in late larvae to adult males. This is consistent with the cell-type specificity and timing of the switch from the *β1-tubulin* to the *β2-tubulin* isoform that occurs in early spermatocytes in third larval instar *Drosophila* (Buttgereit and Renkawitz-Pohl, 1993; Hoyle et al., 1995). The specificity of the *Asβ2tub* promoter function was further verified by linking it to a fluorescent-protein reporter, which showed testis fluorescence specifically in the spermatocytes in transgenic males. The strong expression of the reporter gene, lacking the *Asβ2tub* 3′ UTR, indicated a minimal if any influence of this domain on DsRed transcript expression or stabilization, which differs from its presumed role for *Asβ2tub* transcript in *Drosophila* (Hoyle et al., 1995).
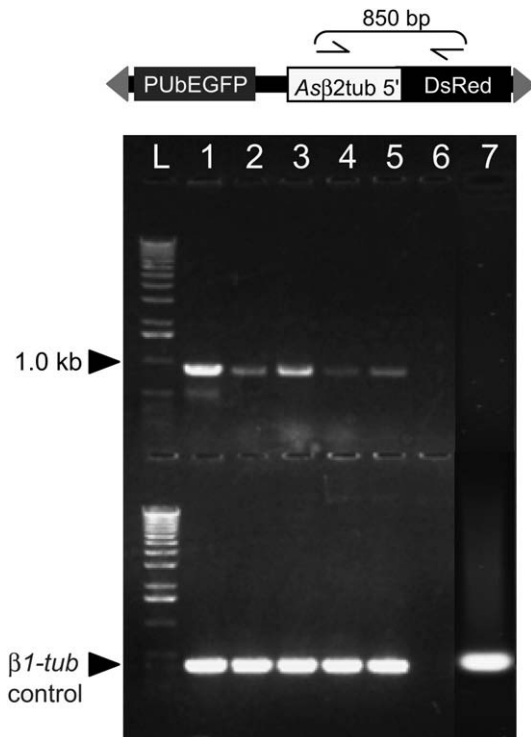
Fig. 6. PCR detection of the Asβ2tub-DsRed.T3 vector sequence in sperm from transgenic males transferred to the abdomens of non-transgenic females after mating. On top is a schematic of the transformation vector (not to scale) showing approximate primer site positions (arrows) for the 850 bp PCR product. Below is an ethidium bromide stained agarose gel showing (top portion) a marker ladder (L), and 850 bp PCR products from DNA extracted from: a transgenic male abdomen (lane 1; positive control), individual abdomens from non-transgenic females mated to transgenic males (lanes 2–5), and an abdomen of a female mated to a non-transgenic male (lane 7; negative control). Negative controls include no DNA template (lane 6) and an abdomen from a non-transgenic female mated to a non-transgenic male (lane 7; from a separate gel). The bottom portion of the gel shows the same samples subjected to PCR using primers for Asβ1tub providing a control for sample DNA. Arrows show the 1.0 kb marker fragment in the top portion and the ~400 bp Asβ1tub product in the lower portion.

The promoter-reporter transgene also provided a means for testis and sperm marking useful for practical application, especially for biological control programs using released insects such as the sterile insect technique. A similar transgene construct resulting in testis-specific fluorescence in *A. stephensi* allowed sexing of mosquito larvae passed through a fluorescent sorter, though this was simplified by their transparent cuticle and relatively small size (Catteruccia et al., 2005). Testis fluorescence could also be detected in *A. suspensa* last instar larvae, and while theoretically usable for sexing, fluorescence was not always visible, possibly obscured by larval fat body, and third instar tephritid larvae are generally too large for fluorescent sorters currently available. A more easily addressed need for marked sperm in biocontrol programs is the ability to determine whether female insects trapped in the field have mated with released males. A current method used in tephritid flies is based on size differences of irradiated and non-irradiated sperm (McInnis, 1993), but this assessment is somewhat subjective, and future transgene-based sterility should eliminate the need for irradiation (Robinson et al., 2004). Our studies showed that, similar to *A. aegypti*, fluorescent-marked sperm could be readily detected in the spermathecae and spermathecal duct of mated females. The presence of these sperm could also be verified unambiguously by PCR amplification of the transgene in individual non-transgenic female abdomens. PCR analysis provides an important secondary verification of mated females, and also for quality control during mass rearing.

The testis-specific β2-tubulin promoter also provides the possibility of inducing male sterility after linkage to cell-autonomous lethal effector genes. These might include cell death genes, toxin subunits, or conditional cell lethal genes (Handler, 2002). It has also been suggested that a germ-line-limited source of transposase in disease vectors could be beneficial in strains having a transposon-drive system (James, 2005). While this is not currently envisioned for tephritid pest control strategies, gene expression limited to the male germ-line adds an important component to our ability to manipulate these insects for functional analysis and practical applications.

Acknowledgements

Appendix. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ibmb.2009.05.004.

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